Exhibit F

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Th1 versus Th2 responses in AIDS

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During the past two years, a simple theory that seeks to explain what causes the progression of HIV-infected individuals to AIDS has been gaining support. The theory holds that HIV-infected people switch from a T-helper type 1 (Th1) to a T-helper type 2 (Th2) state as the disease progresses. However the experimental data do not support the concept that a Th1/Th2 switch occurs in the majority of HIV-infected subjects, although it is conceivable that HIV-infected individuals who mount sustained and chronic Th2-type responses, as a result of allergic disorders and helminthic infestations, may undergo more active HIV replication and therefore progress faster to full-blown disease.

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Introduction

A major advance in understanding the regulation of specific immune responses to infectious agents was the identification of two subpopulations of murine $CD4^+$ T helper (Th) lymphocytes (termed Th1 and Th2), based on their mutually exclusive production of cytokines [1]. Th1 cells produce interleukin (IL)-2, interferon (IFN)-γ and tumour necrosis factor (TNF)-β, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13. Other cytokines, such as IL-3, TNF-α and granulocyte macrophage-colony stimulating factor (GM-CSF) are produced by both Th cell populations. Th1 cells are the principal effectors of cell-mediated immunity against intracellular microbes and of delayed-type hypersensitivity (DTH) reactions, but they also stimulate the production of antibodies of the IgG_{2a} class, which are effective at activating complement and opsonizing antigens for phagocytosis [1]. Thus, Th1 cells trigger phagocyte-mediated host defense and infections with intracellular microbes tend to induce Th1-type responses (Fig. 1). On the other hand, Th2 cells induce the production of IgE and IgG_1 antibodies (via IL-4), favor the growth of mast cells (via IL-3, IL-4, and IL-10), the differentiation and activation of eosinophils (via IL-5), and are capable of inhibiting some macrophage functions (via IL-4 and IL-10) [1]. Therefore, Th2 cells are predominantly involved in phagocyte-independent host defense, e.g. against certain helminths, as well as in the response to common environmental allergens, which is mediated by IgE antibodies and eosinophils (Fig. 1).

Although in humans the expression of some cytokines, such as IL-2, IL-6, IL-10, and IL-13, may be less restricted than in mice, there is now considerable consensus for the existence of $CD4^+$ Th subsets with cytokine patterns and functions that are comparable to murine Th1 and Th2 cells [2••]. Such evidence has been provided both *in vitro* by establishing clones specific for peculiar antigens (e.g. purified protein derivative (PPD) for Th1 and allergens or *Toxocara canis* secretory/excretory antigens for Th2) [2••,3,4] or generated from patients with particular diseases (e.g. Hashimoto's thyroiditis for Th1 and allergic disorders for Th2) [2••,3,4], and *in vivo* by looking at the presence of these cells in the bronchial mucosa of patients with allergic bronchial asthma by *in situ* hybridization [5] or because of the nature of some human diseases (e.g. a clonal proliferation of Th2 cells in a man with the hypereosinophilic syndrome) [6•]. Therefore, it is now clear that several strong immune responses, especially against parasites, are mostly Th1 or Th2 type. There are, however, other $CD4^+$ Th cell phenotypes, Th0 cells, characterized by the production of both Th1- and Th2-type cytokines. Thus, the pattern of the Th cell-mediated specific immune response is not just confined to Th1 and Th2 type: these two cell subsets should be regarded as polarized forms of a much more heterogenous effector response.

The nature of Th1 or Th2 polarizing signals is not yet fully understood. However, the factors that seem to play a role in driving naive $CD4^+$ T cells toward Th1- or Th2-dominated populations are the type and the amount of antigen, the nature of antigen-presenting cells and of their co-stimulants, and the activity of cytokines secreted by other cells in the microenvironment. In both mice and humans, IL-12 (a powerful IFN-γ inducer), produced by macrophages and B lymphocytes, promotes Th1 differentiation [7,8•,9•]. IFN-α and tumour growth factor (TGF)-β produced by macrophages

Abbreviations

DTH—delayed-type hypersensitivity; **GM-CSF**—granulocyte macrophage-colony stimulating factor; **IFN**—interferon; **IL**—interleukin; **NK**—natural killer; **PB**—peripheral blood; **PBMC**—peripheral blood mononuclear cell; **PCR**—polymerase chain reaction; **PHA**—phytohemogluttinin; **PPD**—purified protein derivative; **TCR**—T-cell receptor; **TGF**—tumour growth factor; **Th**-T helper; **TNF**—tumour necrosis factor.

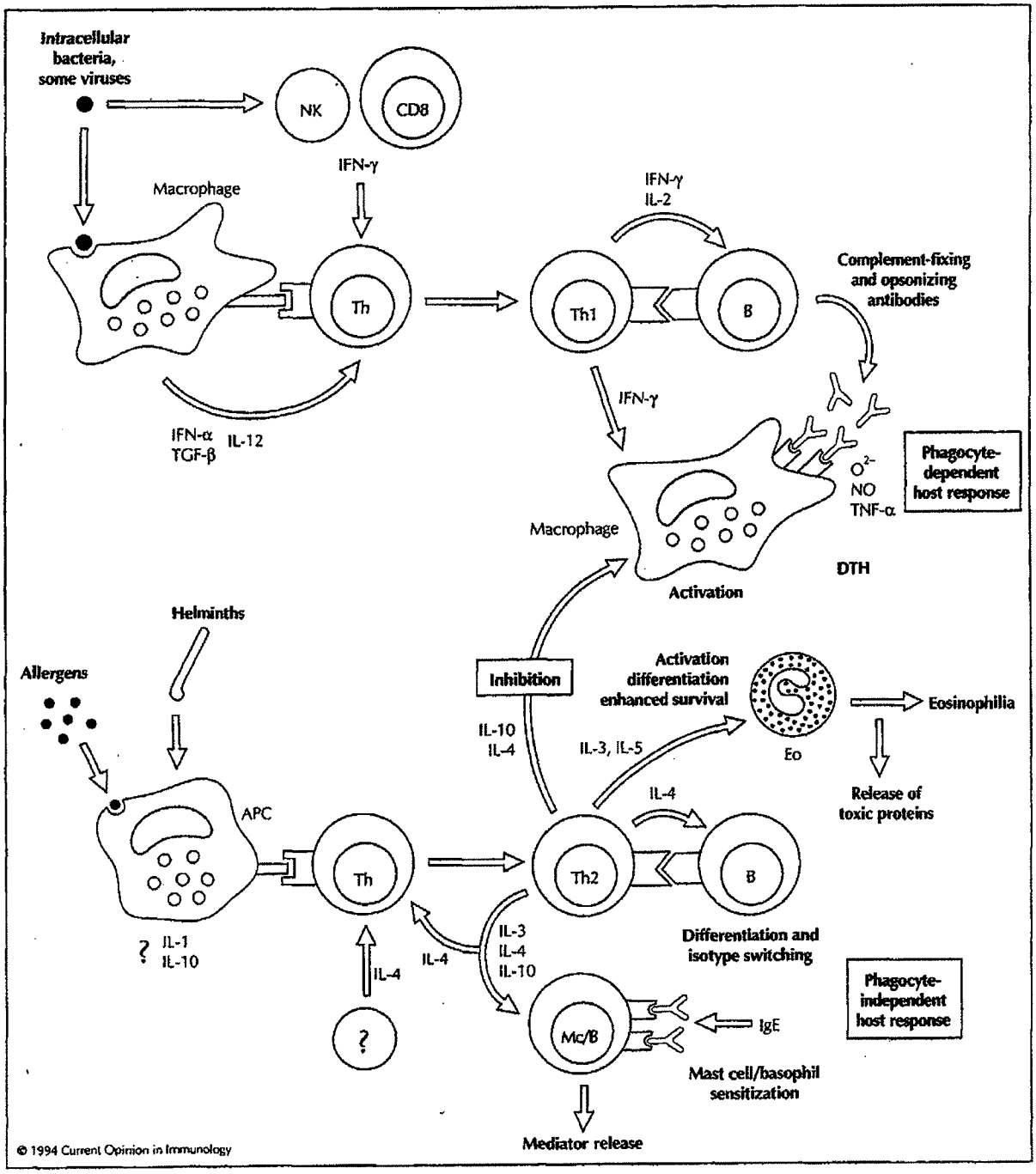

Fig. 1. The two main pathways of specific immunity based on their dependence or independence of phagocyte recruitment in the effector response. Th = CD4+ helper T lymphocyte; CD8+ = CD8+ T lymphocyte; NK = natural killer cell; B = B lymphocyte; DTH = delayed type hypersensitivity; Eo = eosinophil; APC = antigen-presenting cell; Mc/B = cell of the mast cell/basophil lineage. ? = the nature of the cell(s) providing IL-4 at the triad (APC/Ag/Th) recognition level is still unclear.

and IFN-γ produced by natural killer (NK) cells may also contribute, but they seem to play a secondary role in comparison to IL-12 (Fig. 1). On the other hand, IL-4 appears to be the most dominant factor in determining whether naive Th cells become Th2 polarized [10,11]; IL-1 and IL-10 may be less important Th2-inducing co-factors ([12]; R. Manetti, unpublished data). Cells belonging to the mast cell/basophil lineage, CD4−CD8− T-cell receptor (TCR)αβ+ cells and a subset of CD4+ T cells have been suspected to be responsible for the early

IL-4 production [13,14]. However, the source of IL-4 initially required by naive Th cells to develop into the Th2 phenotype still remains unclear.

The 'Th1/Th2 switch' theory in HIV infection: facts and implications

One of the most dramatic effects of HIV infection on the immune system is the severe depletion of $CD4^+$ T cells in later stages of infection. In a proportion of HIV-infected patients, however, immunity and particularly T-cell function is affected despite adequate numbers of $CD4^+$ T cells [15••]. Thus, peripheral blood mononuclear cells (PBMCs) from asymptomatic, HIV-infected individuals can exhibit defects of *in vitro* antigen-stimulated T-cell proliferation or IL-2 production. In contrast, enhanced B-cell responses are common, as revealed by hypergammaglobulinemia and spontaneous IgG production *in vitro* [15••]. The finding that T-cell proliferation and IL-2 production decline in HIV-infected individuals, while B-cell activity is increased, has led to the speculation that a switch from the Th1 to the Th2 cytokine phenotype may be important in the pathogenesis of the disease progression in HIV infection [16•]. This hypothesis was based on two distinct observations. Firstly, Clerici *et al.* [17] found that individuals exposed to HIV, who tested negative for the virus and did not seroconvert, showed evidence of HIV-specific cell-mediated immunity as measured by IL-2 production by PBMC in response to HIV envelope peptides. This does not prove, however, resistance to infection, as the HIV peptide-induced IL-2 production may merely reflect exposure to single HIV components and not the viable virus. Secondly, in another series of experiments, Clerici *et al.* [18] demonstrated that 50% of asymptomatic, HIV-infected patients showed a gradual shift from a predominance of Th1- to Th2-type responses in the course of HIV infection. Loss of initially good IL-2 responses to soluble antigens or HIV peptides was often accompanied by increased mitogen-induced IL-4 production. However, these findings are not fully convincing. First, in the human system, IL-2 production is not strictly limited to Th1 cells and production of the most representative Th1 cytokine, i.e. IFN-γ, was not assessed. Moreover, IL-2 production was assessed in response to either soluble antigens or HIV peptides, whereas mitogen-stimulation was used to assess IL-4 production. Third, a biological assay was used to measure IL-4, which may not guarantee specificity. Finally, increased IL-4 production was found only in a small proportion of HIV-infected subjects, showing a defective response to recall antigens but not to allo-antigens or phytohemagluttinin (PHA), whereas PBMC from all other HIV-infected individuals show no increase or even a decrease in IL-4 production [16•]. More recently, the same authors have shown that both defective T-cell proliferation and IL-2 production to influenza virus and synthetic peptides of HIV envelope could be reversed when cultures were stimulated in the presence of IL-12 [19]. Finally, they have observed increases in IL-10 mRNA and mitogen-stimulated IL-10 production in virtually all HIV-infected individuals, especially the more severely immunodeficient patients. Addition of anti-IL-10 antibody also restored the proliferative response to HIV-specific antigens, such as the envelope peptides [20].

If correct, the Th1/Th2 switch hypothesis would have major practical implications for developing AIDS vaccines and other forms of therapies. It is generally believed that in order to provide optimal protection, vaccination against HIV should induce both a strong neutralizing antibody response and a strong cell- mediated immune response. Based on the above mentioned findings, however, it has been suggested that the induction of a strong antibody Th2-type response would not be desirable, as it may suppress the protective cell-mediated Th1-type response due to the effects of cross-regulatory (IL-4 and IL-10) cytokines [21•]. Thus, vaccination against HIV capable of inducing a selective cell-mediated, Th1-type response against HIV should preferentially be developed.

It is very difficult to demonstrate a switch from a Th1 to Th2 state, as Th1 and Th2 markers are currently not available. Therefore, in order to determine the validity of the Th1/Th2 switch theory, different experimental approaches have been pursued (Table 1). Analysis of the constitutive expression of a group of cytokines including IL-2, IL-4, IL-10, and IFN-γ in unfractionated mononuclear cells and sorted $CD4^+$ and $CD8^+$ T cells isolated from both peripheral blood (PB) and lymph nodes from the same HIV-infected individuals was unsuccessful. IL-4 was not expressed in any cell subset in either PB or lymph node, and $CD4^-$ cells were predominantly responsible for the expression of IFN-γ and IL-10 [22••]. In another study, elevated IFN-γ and decreased IL-2 gene expression was reported, but there was no significant change in IL-4 mRNA levels [23]. Thus, there is no evidence of a persistent Th2-type response in PB or lymph nodes from HIV-infected individuals. The search for changes in the cytokine profile of cells stimulated *in vitro* has also been disappointing. Experiments performed in our laboratory failed to demonstrate enhanced production of IL-4 and/or IL-10 by PBMCs from HIV-infected patients, in any phase of HIV infection, in comparison with HIV-seronegative controls [24,25••]. Similar data have recently been obtained by another group (J Chehimi, personal communication). One possible explanation for these discrepancies may be the different categorizations of HIV-infected individuals, since we did not subdivide our patients on the basis of their antigen-reactivity. This possibility is, however, unlikely because in no case did we find enhanced IL-4 production by the PBMCs of our patients with more than 500 $CD4^+$ T cells μl^{-1}, which included all of the different antigen-reactive groups. Nor is the discrepancy due to the lower sensitivity of ELISA compared with the sensitivity of the biological assay, as the ELISA is capable of detecting IL-4 concentrations as low as 5–10 $pgml^{-1}$. On the other hand, our findings are not surprising as the levels of IL-4 detected in the supernatants of PBMC or purified $CD4^+$ T cells stim-

Table 1. Experimental approaches used to determine the validity of the 'Th1/Th2 switch' hypothesis.

Assay system	Cells involved	Changes in cytokine production: IFN-γ	Changes in cytokine production: IL-4	References
Cells stimulated *in vitro*				
PBMC	T cells & non-T cells	↓[a]	↑[b]	[16•,18,20]
PBMC	T cells & non-T cells	↓	↓	[24,25••]
Polyclonal CD4+T-cell lines	All CD4+T cells	↓	↓	[24]
Cloned T cells				
PHA-induced (all T cells)	Naive and memory T cells	=[c]	↓[d]	[24,25••]
PHA-induced (CD45RO+T cells)	Memory T cells	=	↑	A Meyaard *et al.*, unpublished data
Skin-derived, IL-2-expanded	*In vivo* activated memory T cells	=	↑	[24,25••]
Antigen-specific	*In vitro* activated memory T cells	=	↑	[24,25••]
		mRNA by PCR		
Unstimulated cells				
PBMC	*In vivo* responding T cells & non-T cells	+[e]	–[f]	[22••]
PBMC	*In vivo* responding T cells & non-T cells	++[g]	–	[23]
Purified CD4+T cells	*In vivo* responding CD4+T cells	+	–	[22••]
Purified CD4+T cells	*In vivo* responding	++	–	[23]

[a] = decrease; [b] = increase; [c] = no difference; [d] = decrease only in patients with advanced HIV infection; [e] = expressed; [f] = not expressed; [g] = hyperexpressed. Analysis of the constitutive cytokine expression in lymphoid tissues of HIV-infected individuals revealed high levels of IFN-γ, and low or no expression of IL-2 and IL-4. The results of studies on cytokine production in response to cell stimulation *in vitro* varied according to the model employed. Increased production of IL-4 and IL-10 by mitogen-stimulated PBMC from HIV-infected individuals [16•,18,20] was not confirmed ([24,25••]; J Chehimi, personal communication; A Vyakarnam, personal communication; P Galanaud, personal communication). Studies at the clonal level revealed no increase in IL-4 production, and even a decrease of IL-4 producing cells in the advanced phases of HIV infection, when all CD4+T cells (naive, memory, resting and activated) were expanded. Only a shift from Th1 to Th0 profile of cytokine secretion was observed when *in vivo* or *in vitro* activated memory CD4+T cells were clonally expanded.

ulated in short-term cultures are generally low even in pathological conditions characterized by enhanced IL-4 production, such as allergic disorders and helminthic infestations. To circumvent this problem, cytokine production was determined in a large panel of $CD4^+$ T-cell clones generated from the PB of nine HIV-infected and nine HIV-seronegative individuals by a high efficiency cloning technique, which allows the expansion of virtually every T cell. The proportions of $CD4^+$ T-cell clones which produced IFN-γ were comparable in the two groups of subjects, whereas the proportions of clones which produced IL-4 and IL-5 were significantly lower in HIV-infected patients, especially those showing low levels of circulating $CD4^+$ T cells [24,25••]. When T-cell clones were generated from selected populations, however, such as $CD45RO^+$ (memory) PB T cells (L Meyaard, S Otto, IPM Keer, AW van Lier, F Miedema, Abstract 79, 3rd International Conference on Cytokines, Florence, Italy, March 1994), antigen (PPD or *Toxoplasma gondi*)-specific PB T cells [24, 25] or IL-2-expanded skin-derived T cells [24,25••], an increase was observed in the proportion of cells producing both Th1- and Th2-type cytokines. Taken together, these results show no evidence of a dichotomy of Th1 and Th2 predominance in lymphoid tissue of HIV-infected individuals, at most they indicate that a Th1/Th2 shift in a proportion of memory $CD4^+$ T cells may occur in some patients. Finally they demonstrate a selective depletion of $CD4^+$ T cells that produce Th2 cytokines in the advanced phases of HIV infection.

The mechanisms responsible for this phenomenon (i.e. Th1 to Th0 switch) are still unclear. One explanation may be that it reflects the altered balance of cytokines produced by HIV-infected macrophages. As mentioned above, cytokines released by macrophages play a critical role in determining the differentiation of T cells into distinct functional phenotypes at the time of antigen presentation [7,8•,9•,10,11]. It is worth noting that the production of IL-12 and IFN-α (both Th1-inducing agents) is defective in HIV-infected patients [25••,26,27••], whereas the production of IL-1, IL-6,

IL-10, TNF-α and GM-CSF is normal or increased [28]. Therefore, the combined IL-12 and IFN-α defective production by HIV-infected macrophages may favor the enhanced expression of Th2-type cytokines even in response to antigens usually evoking Th1 responses.

Another possibility may be the emergence, in at least some patients, of an IL-4-producing cell type which creates microenvironmental conditions more suitable for the differentiation of antigen-stimulated T cells into a less restricted Th1 profile. In two HIV-infected patients suffering from recurrent skin and sinopulmonary infections, pruritus, high serum IgE levels and eosinophilia (the so called Job's-like syndrome), we have recently shown a switch of cytolytic $CD8^+$ T cells to cells which exhibit reduced cytolytic potential and a clear-cut Th2 cytokine profile [29]. These $CD8^+$ Th2-like cells are reminiscent of murine $CD4^-CD8^-$ cells resulting from switching of $CD8^+$ T cells incubated *in vitro* with high IL-4 concentrations [30•]. It is interesting that both patients had virtually no circulating $CD4^+$ T cells (i.e. less than 50 cells μl^{-1}), but showed high proportions of circulating $CD4^-CD8^-TCR\alpha\beta^+$ cells which produced high concentrations of IL-4 [29], and at least some of which were $CD38^+$. These cells may be equivalent to $CD38^+CD4^-CD8^-TCR\alpha\beta^+$ cells, the earliest cells capable of producing IL-4 in the mouse adult thymus [31]. Interestingly, the murine $CD38^+CD4^-CD8^-TCR\alpha\beta^+$ cells appear to be related to $CD38^+CD8^+$ thymocytes which, in turn, are reminiscent of $CD38^+CD8^+$ T-cell populations that increase early after infection with HIV and usually continue to increase over time [32]. Whether all these cell types are indeed linked by a unique pathophysiological thread remains to be established.

Preferential replication of HIV in Th2-like cells rather than induction of a Th1/Th2 switch

In addition to the possibility that HIV favors an enhanced production of Th2-type cytokines even in response to Th1-inducing antigens, such as components of intracellular parasites, at least in a proportion of infected subjects, some of the above mentioned studies also suggest a preferential depletion of $CD4^+$ Th2-type cells in the advanced phases of HIV infection. First, the proportions of PHA-induced T-cell clones induced to produce IL-4 and IL-5 were significantly reduced in HIV-infected patients in comparison with controls, because of the preferential depletion of Th0 and Th2 cells in the patients with low numbers of circulating $CD4^+$ T cells (i.e. lower than 200 μl^{-1}). This depletion was associated with a significant decrease in the cloning efficiency [24,25••], suggesting either direct virus-induced killing or programmed death of these cells during the cloning procedure. Secondly, constitutive expression of IL-4 mRNA was barely, if at all detectable in PB or lymph nodes at any stage of the disease [22••]. Finally and most importantly, studies performed both in our [24,25••] and in another laboratory have shown a differential ability of Th1 and Th2 $CD4^+$ T-cell clones to support viral replication *in vitro*. In our study, 52 $CD4^+$ T-cell clones generated from HIV-seronegative individuals were infected *in vitro* with HIV and three weeks later infection was assessed by both amplification with the polymerase chain reaction (PCR) and measurement of p24 antigen production in the culture supernatants. HIV-infected clones showed the presence of DNA provirus by PCR; however, all the 11 Th2 and 22 out of 33 Th0 clones, but none of the 8 Th1 clones, produced p24 antigen in their supernatant. Accordingly, Th1 but not Th2 clones specific for HIV-1 gag p24 inhibit HIV replication (A Vyarkanam, Abstract 81, 3rd International Conference on Cytokines, Florence, Italy, March 1994). These results suggest that, at least *in vitro*, HIV preferentially replicates in T cells that produce Th2-type cytokines. The higher resistance of Th1 clones to HIV replication did not reflect their lytic activity for virus-producing clones, as the number of T-cell blasts from HIV-infected Th1 clones and their non-infected counterparts remained comparable. Moreover, it was not simply related to their ability to produce IFN-γ, as the majority of Th0 clones also efficiently supported viral replication. In agreement with previously reported results [32], we have recently shown that $CD4^+$ T cells from some HIV-infected patients could be triggered to produce virus simply by depleting from PBMC suspensions of $CD8^+$ T cells. More importantly, at least in a proportion of HIV-infected patients, incubation with anti-IFN-γ or anti-IFN-γ receptor antibody was sufficient to trigger spontaneous p24 antigen production (virus production) in purified PB $CD4^+$ T cells [25••]. As HIV suppression by $CD8^+$ T cells is mediated by presently uncharacterized soluble factors [32], and taking into account the fact that $CD4^+$ Th1 cells exhibit a cytokine profile similar to $CD8^+$ T cells [1,2••], it is reasonable to suggest that IFN-γ, possibly in concert with other soluble factor(s) released by both $CD8^+$ and $CD4^+$ Th1 cells, plays an important role in protecting $CD4^+$ Th1 cells from active viral replication. On the other hand, it is also possible that the protective effects of IFN-γ may be substantially compromised in Th0 clones which efficiently support HIV replication by the co-production of counter-regulatory cytokines, such as IL-4 and IL-10.

The possibility that Th2-like cells may allow higher HIV replication, thus favoring their own destruction and further spread of virus, is supported by the recent observation that HIV-infected subjects showing elevated IgE serum levels at the time of virus infection progress more rapidly towards both depletion of circulating $CD4^+$ T cells and development of full-blown disease than HIV-infected subjects who exhibit low or undetectable IgE serum levels at the time of serodiagnosis [24]. It may also account for the faster progression to AIDS of HIV-infected subjects living in geographical areas highly infested by helminth parasites (Z Bentwich, personal communication). Thus, it is reasonable to suggest that progression of the disease in HIV-infected individuals does not result from a Th1/Th2 switch, but may be favored by active viral replication in $CD4^+$ T cells stimulated to preferen-

tially produce Th2 cytokines by common environmental allergens or helminthic infections.

Conclusions

During the past few years, a very simple theory that seeks to explain what causes the relentless and ultimately fatal decline of AIDS patients has received considerable attention. The theory holds that HIV-infected people switch from a Th1 to a Th2 state as the disease progresses. If correct, the theory could have important implications both in vaccine development for prevention of HIV infection and in treatment of HIV disease. As the Th1 state is mainly responsible for cell-mediated responses, whereas the Th2 state preferentially results in antibody responses, it was inferred that the goal of immunization to prevent or control HIV infection should be activation of the Th1-type cell-mediated, rather than the Th2-type antibody-mediated, arm of the immune system [21•]. More recently, the Th1/Th2 theory has gained further support from the observation that IL-4 gene-targeted mice lacking Th2 responses do not develop murine AIDS (MAIDS) [33•].

Although suggestive in principle, the Th1/Th2 switch theory can be faulted on several grounds. First, as has already been stressed [34], it is not correct to designate antibody responses in general as being Th2 responses. Although production of IgE and IgG_4 (or IgG_1 in mice) appears to be restricted to Th2 cells, production of other immunoglobulin isotypes, including highly protective complement fixing and opsonizing antibodies, is mainly due to Th1 cells in both mice and humans. Secondly, the finding that the lack of IL-4 gene accounts for resistance to MAIDS has recently been challenged; the association with another as yet unknown gene product is required [35]. The genetics of susceptibility to MAIDS virus certainly appears to be rather complex and not solely dependent on IL-4. Moreover, the results of different experimental approaches at the level of both unfractionated PBMC and cloned T cells do not fully support the occurrence of Th2-dominated responses in any phase of HIV infection. At the most, the results of some studies suggest the possibility of a shifting in a proportion of memory T cells from the Th1 to the Th0 profile of cytokine secretion even in response to Th1-inducing antigens [24,25••]. Finally, analysis of the constitutive expression of cytokines in PB and lymph nodes from HIV-infected individuals failed to demonstrate an *in vivo* switch from Th1 to Th2 cytokine phenotype as HIV disease progresses [22••,23].

Although induction of a Th2 state is clearly not a general phenomenon in HIV infection, both conceptual and experimental evidence suggest that HIV-infected subjects, who mount sustained and chronic Th2-type responses either because of their genetic background (e.g. people with atopic allergy) or the environment (e.g. individuals living in helminth-infested areas), may undergo more active HIV replication and therefore progress faster to AIDS. Thus, the understanding of why Th2 cells are more efficient supporters of virus replication than Th1 cells may have potentially important implications in the design of therapeutic strategies.

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